Purification of Rubella Virus Particles

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SUMMARY

A procedure for the purification of rubella virus from infected suspensions of BHK21 cells resulted in preparations containing 1 to $3 \times 10^{10}$ p.f.u./ml. In sucrose gradient centrifugation the area of maximal infectivity (buoyant density of 1.175 g./ml.) coincided with peaks in haemagglutinating activity, and in $E_{260}$. It also coincided with presence of virus-like particles, and a sharp band visible by naked eye. Specific activities of the order $30 \times 10^6$ p.f.u./mg protein, $10^6$ HAU/mg protein, and 5 to $10 \times 10^5$ p.f.u./HAU were achieved.

Glutaraldehyde-fixed negatively stained rubella virus preparations showed round particles with rough surfaces measuring 50 to 73 nm. (average 61 nm.) in diameter. Unfixed viruses had a greater size variation, 55 to 89 nm. (average 74 nm.) in diameter, apparently due to deformability and fragility of the particles. Spontaneous and deoxycholate-induced breakdown of the particles showed rupture of the envelope but revealed no characteristic inner structure.

INTRODUCTION

The biophysical and biochemical characterization of rubella has been difficult largely because high titres are difficult to obtain, and because rubella virus is sensitive to various physical and chemical conditions. Sensitivity of particles to lipid solvents and resistance to inhibition of growth by thymidine analogues are indirect evidence of the presence, respectively, of lipid and RNA in the virion. The variations in average particle size (50 to 70 nm.), and buoyant density in sucrose gradients (1.05 to 1.20 g./cm$^3$) (Best et al. 1967; Holmes et al. 1968; Russell, Selzer & Goetze, 1967; Vaheri, Sedwick & Plotkin, 1967; Magnusson & Skaaret, 1967; Cusumano, 1966; Veronelli & Maassab, 1965), reported for rubella virus are striking. In its electron microscopic appearance (Holmes & Warburton, 1967), relative resistance to Actinomycin D (Maes et al. 1966), association with eosinophilic intracytoplasmic inclusions in cell cultures (Maassab & Veronelli, 1966), Ca$^{2+}$-dependent haemagglutination at an acid pH optimum (Stewart et al. 1967) and production of both soluble and particle-associated complement-fixing antigens in cell cultures (Schmidt, Lennette & Gee, 1966; Furukawa, Vaheri & Plotkin, 1967a) rubella virus seems to share characteristics with some arbo-, paramyxo- and leucosis viruses, and therefore it cannot be properly classified. The large-scale production of high concentration of rubella virus in suspended BHK21/13S cells (Vaheri et al. 1965, 1967) enabled us to study the purification and biophysical characterization of the rubella virion.
METHODS

**Virus production.** The RA 27/3 strain of rubella virus was used after it had undergone 4 passages in WI-38 cells and 32–35 passages in BHK21 cells (Vaheri et al. 1967) at 34 ° in 'BHK21-medium' (enriched Eagle's basal medium) supplemented with 10 % tryptose phosphate broth (Difco) and 5 % newborn-calf serum (heat-inactivated at 50 ° for 30 min.). The rubella virus containing supernatant medium harvested by low-speed centrifugation was neutralized to about pH 7.3 by NaHCO₃, and then stored at −70 ° or processed further immediately. In practice, at m.o.i. = 10 p.f.u./cell the harvest (30 to 40 hr) gave 10⁷.5 to 10⁸.5 p.f.u./ml.

**Plaque assay.** The present modification of our previous BHK21/WI-2 cell procedure (Vaheri et al. 1967) has the following essential features: (1) Virus diluent was Basal Medium Eagle (Diploid) containing 0.2 % bovine serum albumin but with the plaque reduction tests 2 % inactivated newborn calf serum was employed instead of albumin. (2) Overlays were composed of 1 vol. of 2 % sodium carboxymethyl-cellulose (Type 7 HP, Hercules Powder Co., Wilmington, Del.) and 3 vol. of Basal Medium Eagle (Diploid) (Grand Island Co., Grand Island, N.Y.) containing only 0.3 g/l. of NaHCO₃. (3) Incubation was for 6 to 7 days at +34 ° in 3 ° CO₂/97 ° air. (4) Clear 2 to 3 mm. rubella virus plaques were regularly obtained under these carefully standardized conditions. They were stained with crystal violet.

**Haemagglutination (HA) test** has been described (Stewart et al. 1967). The end point was taken to be partial agglutination of red cells = 1 HAU.

**Protein concentration and optical density.** Protein was assayed by the method of Lowry et al. (1951) and optical density and u.v.-absorption spectra were determined in a Beckman Type DU or Type DB or Zeiss Type PM QII/M 4 QII spectrophotometer.

**Electron microscopy.** Preparations for electron microscopy were made on carbon coated grids using 1 or 2 % phosphotungstic acid as negative stain. Electron micrographs were taken with Siemens Elmiskop IA microscope at original magnifications from 10,000 to 40,000. The diameter values of rubella virus were based on at least 100 particles.

**Deoxycholate treatment.** One drop of the specimen was put on the grid, allowed to settle for 30 sec. and dried with filter paper. Immediately thereafter a drop of 0.01 % sodium deoxycholate was applied and allowed to act for 20 to 120 sec. The specimen was then rinsed in distilled water and negatively stained.

**Prefixation with glutaraldehyde.** Glutaraldehyde was added to the virus-containing tissue culture fluid to a final concentration of 0.5 %; after fixation for 1 hr at +4 ° the material was dialysed overnight against tris + EDTA solution (0.05 m-tris + HCl buffer, 0.001 m-EDTA, pH 7.3).

**Virus identification and sterility tests.** The purified virus in the infectivity peaks of gradients was identified as rubella virus by haemagglutination inhibition and by neutralization in BHK21/WI-2 plaque reduction tests against pairs of sera (i.e. a negative acute stage and a positive convalescent stage serum sample from patients with verified rubella). Mycoplasma could be isolated from none of the rubella virus or BHK21-cell preparations on Hayflick's (1965) nutrient agar within 14 days.

**EDTA treatment.** In order to remove the nonspecific haemagglutination inhibitor, 0.03 m-EDTA, neutralized with tris, was added and the medium incubated for at least 1 hr at +4 ° (Furukawa et al. 1967b) before the next step.
**Rubella virus purification**

*High-speed centrifugation.* The virus was pelleted by centrifugation at 40,000 g (Spinco Rotor 30) at +4° for 2 hr. The pellet was rinsed carefully with tris+EDTA, resuspended in the same buffer by means of needle and syringe and sonicated for 2 min. just before gradient centrifugation.

*Gradient centrifugation.* About 0.5 ml. of sample was layered on 4.2 ml. of a linear 5 to 50 % (w/w) sucrose gradient in tris+EDTA buffer and was centrifuged at 30,000 rev./min. at +4° for 2 hr in the SW65 rotor of a Spinco centrifuge. The fractions were collected by dripping from a hole punctured in the tube bottom. Their densities were determined in an Opton type 56618 Abbé-refractometer.

*Ultrasonic treatment.* A Raytheon 10 kcyc. Sonic oscillator Model DF-101 was used at 115 V 50 Hz with the material in plastic tubes immersed in ice water.

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**RESULTS**

The best yields of largely purified rubella virus have been obtained by the procedure shown schematically in Fig. 1. Vacuum dialysis was selected from a number of methods tested for 'volume concentration'. Rubella virus was precipitable with 7.5 % polyethylene glycol (Kanarek & Tribe, 1967), 0.02 M-zinc acetate (Vaheri *et al.* 1967), 50 % saturated (NH₄)₂SO₄ or 30 to 40 % ice-cold methanol, but these methods were no good for low-speed sedimentation from large volumes and gave only a 30 to 60 % recovery of infectivity.
Density gradient centrifugation proved to be very effective in removing impurities from the virus preparation (Table 1). Essentially all rubella virus infectivity was recovered in one peak at a density of 1.17 g./ml. (Table 1, Fig. 2). This area of maximal p.f.u. coincided with a sharp band, visible by naked eye particularly under dark-field illumination, with peaks in $E_{260}$ and $E_{280}$, with a peak in haemagglutination and with the presence of virus-like particles by electron-microscopy (Fig. 2). None of these characteristics was detected in identically treated control preparations.

![Graph](attachment:image.png)

**Fig. 2.** Centrifugation of partially purified rubella virus preparation in a linear 5 to 50% (w/w) sucrose gradient for 2 hr at 30,000 rev./min. in the Spinco SW65 rotor. • — •, p.f.u.; O—O, HAU; △ — △, $E_{260}$; □ — □, specific gravity.

**Properties of RV preparations after purification**

From 1000 ml. volumes of starting material, preparations containing around $10^{10}$ p.f.u./ml. were obtained (see Table 1). Specific activities of the order $3 \times 10^7$ p.f.u./µg. of protein, $10^8$ HAU per µg. of protein, $E_{260} = 5$ units per $10^{10}$ p.f.u. were repeatedly achieved. The p.f.u./HAU ratio has varied in different preparations between 5 to $10 \times 10^4$. In u.v. absorption spectra a peak was seen in the 240 to 250 nm. region.

Rubella virus could not be further purified by rerunning the peak in a second identical sucrose gradient centrifugation. In all of three such attempts from an input of $10^{10}$ p.f.u. only 0.1 to 1.0% was recovered and the $E_{260}$ peak flattened to cover the entire gradient fractions.

In rate zonal sedimentation experiments (5 to 15% sucrose, 20 to 30 min. SW65,
Table I. Purification procedure

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Volume (ml.)</th>
<th>p.f.u./ml.</th>
<th>HAU/0.05 ml.</th>
<th>Protein µg./ml.</th>
<th>Total p.f.u.</th>
<th>Specific activity/µg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cell supernatant fluid</td>
<td>1000</td>
<td>$4.2 \times 10^5$</td>
<td>5</td>
<td>3860</td>
<td>$4.2 \times 10^{10}$</td>
<td>$1.09 \times 10^4$</td>
</tr>
<tr>
<td>2. 10,000 g 15 min. supernatant fluid</td>
<td>1000</td>
<td>$3.8 \times 10^5$</td>
<td>5</td>
<td>—</td>
<td>$3.8 \times 10^{10}$</td>
<td>—</td>
</tr>
<tr>
<td>3. Vacuum dialysis</td>
<td>33</td>
<td>$1.2 \times 10^6$</td>
<td>50</td>
<td>—</td>
<td>$3.7 \times 10^{10}$</td>
<td>—</td>
</tr>
<tr>
<td>4. EDTA treatment</td>
<td>35</td>
<td>—</td>
<td>64</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5. 40,000 g 2 hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Supernatant fluid</td>
<td>35</td>
<td>$1.6 \times 10^6$</td>
<td>2.5</td>
<td>—</td>
<td>$0.6 \times 10^{10}$</td>
<td>—</td>
</tr>
<tr>
<td>(b) Resuspended pellet</td>
<td>0.5</td>
<td>$2.2 \times 10^{10}$</td>
<td>25,600</td>
<td>7100</td>
<td>$1.1 \times 10^{10}$</td>
<td>$3.1 \times 10^6$</td>
</tr>
<tr>
<td>6. Sucrose gradient peak fraction</td>
<td>0.4</td>
<td>$1.7 \times 10^{10}$</td>
<td>32,000</td>
<td>475</td>
<td>$0.7 \times 10^{10}$</td>
<td>$35.8 \times 10^6$</td>
</tr>
</tbody>
</table>
20,000 rev./min.) broad zones of p.f.u. or haemagglutinin were obtained in spite of the use of EDTA-treated and sonic-treated preparations. Electron microscopy of the fractions revealed that this was due to aggregation of virus particles.

Electron microscopy

Preparations resulting from step 6 of the purification procedure contained virus-like particles, 55 to 89 nm. (average 74 nm.) in diameter and small amounts of amorphous and particulate impurities (Pl. I A). At higher magnification the particles (Pl. I B) showed no symmetry in surface structure but seemed to have randomly arranged projections of approximately 50 Å, giving the particles fuzzy contours. The particles were pleomorphic in shape and size, which seemed partly to be due to the deformability ('softness') and the fragility of the particles. No helical structures could be observed in association with the disrupted particles, nor could any definite nucleoids be detected.

When treated with deoxycholate the particles began to disintegrate within 20 sec. and they were totally broken after a 2 min. treatment (Pl. I C). First, the envelope disrupted, allowing the negative stain to penetrate into the particle, and then it opened completely. As with the spontaneously disrupted particles, these showed no symmetrical inner structure.

To avoid deformity and disintegration of the purified rubella virus preparations, some preparations were prefixed with glutaraldehyde. After the standard purification procedure from 100 ml. of starting material, a peak of haemagglutinin—37·5 HAU/0·05 ml.—at 1·17 g./ml. coincided with aggregates of particles found in electron microscopy. These particles had a mean diameter of 61 nm. (range 50 to 73 nm.), which was over 10 nm. less than the unfixed particles and equalled the size of sectioned rubella virus particles (59 nm.) in BHK 21 cells (see von Bonsdorff & Vaheri, 1969).

In control preparations, no particles were formed corresponding either to the rubella virus structure presented here or to the latent hamster virus (Bernhard & Tournier, 1964). Special attempts, including differential centrifugation of cell extracts, were made to search for the hamster virus which has occasionally been seen intracellularly in sectioned BHK 21/13 S cells.

DISCUSSION

The purification procedure herein described for rubella virus achieved a concentration factor of 2500 and gave a 3000-fold increase in specific activity (p.f.u./μg. protein). The infectivity in the purified rubella virus preparation, containing about 3 × 10⁷ p.f.u./μg. protein, represents, however, only 15% of the total infectivity in the starting material. The results (Table I) showed that by far the greatest loss followed the high-speed centrifugation step where only 25% of the input p.f.u. were recovered. Although this might partly have been due to aggregation, previous studies on the 'degenerative' effect of pelleting (Cusumano, 1966) and the present electron microscopic observations on the ready distortion of rubella virus particles suggest the harmful effect of pelleting. Other gentler methods, such as centrifugation on a dense 'cushion' of sucrose or salt or continuous zonal centrifugation, may offer advantages.

Only particle-associated HA activity of rubella virus was detected. The observed HAU/p.f.u. ratio of around 5 to 10 × 10⁵ is comparable to that generally seen in
Morphology of the rubella virus. A. Virus particles purified by the described procedure. Negatively stained. B. Same in higher magnification. C. Deoxycholate treated rubella virus particles.

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myxoviruses. The mean size of the rubella virion is in our opinion 60 nm. This value is based on sectioned material and glutaraldehyde prefixed negatively stained material. The larger mean size value observed in unfixed negatively stained preparations was apparently due to the deformability of the virion which flattened the virus on the grid. All results seem to indicate that considerable heterogeneity of size and shape is a characteristic of rubella virions. Structurally the rubella virus, on the basis of the present results, cannot be definitely classified. The size and surface structure, both in negatively stained and sectioned material, has a close resemblance to that of some arboviruses, e.g. Semliki Forest virus (Calberg-Bacq & Osterrieth, 1966). On the other hand, viruses of the avian and murine leucosis group, although larger in size, are, with present knowledge, structurally indistinguishable from rubella virus. The classification of rubella virus is at present hazardous and must await further information on the inner structure, symmetry characteristics and size of rubella virus RNA.

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REFERENCES


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